then the coloring reaction was monitored at O.D. (405 nm) in a plate reader.

Figure 2 shows the influence of an influenza vaccine, comprising attenuated cholera toxin prepared in Example 2 as the adjuvant, on the production of anti-influenza HA IgA antibody by the secondary response in the nasal wash. When the adjuvant-free vaccine was administered intranasally, the titer of anti-HA-IgA antibody was low. On the other hand, it was observed that the titer of anti-HA-IgA antibody in the nasal wash was markedly elevated in the group subjected to primary and secondary inoculation of vaccine containing attenuated cholera toxin. The titer of antibody is comparable to that of the control in which the same dose of natural cholera toxin was used.

Figure 3 shows the influence on the production of anti-HA IgG antibody in the serum in the above-described test. The attenuated toxin elevated 15 times the titer of HA antibody in the serum as compared with that with the adjuvant-free vaccine. However, the antibody titer was about half of that with natural cholera toxin used as a control. These results show that the attenuated cholera toxin is useful as the adjuvant for vaccination, for example, when the local immunity is required to be enhanced.

Example 4 - Adjuvant activity of highly attenuated toxin:

A variety of attenuated cholera toxins, of which residual toxic activities were all at least one-two thousandth of that of the natural one, were used as the adjuvants. The same experiment as in Example 2 was repeated several times to assay the titer of anti-HA-IgA in the nasal wash and titer of IgG antibody in the blood of mice. The antibody titers determined in each assay were converted to relative values to those observed when the same amounts of natural cholera toxin were use as positive controls. The relative value is indicated as the ordinate axis and the attenuation rate of the toxin (relative residual activity to the natural one) was indicated as the abscissa axis (Figure 4). It is obvious that the majority of attenuated toxins, of which residual toxic activities are at least one-two thousandth that of the natural one (one-two thousandth $\fallingdotseq 4^{-5\ 46}$), exhibit high levels of antibody production-enhancing activity comparable to that of the

same amount of natural cholera toxin. In particular, it was verified that the attenuated toxins, of which residual toxic activities are reduced to at least one-two thousandth that of the natural one, show comparable or higher efficacy relative to that of original toxin in regard to the enhancement of mucous membrane IgA specific to the antigen by intranasal inoculation.

Example 5 - Use of dual adjuvant:

A cholera toxin attenuated by the formalin treatment (having a residual toxic activity of about 1/1048000 that of the natural one in Y-1 cell morphologic transformation test) and *E. coli* heat-labile toxin B subunit (untreated), which toxic activity had previously been confirmed to be weak, was used as the adjuvant together. This experiment was carried out in the same manner as in Example 3. The cellular immune response to influenza virus was evaluated by the mouse foot swelling test. The result is shown in Figure 5. When 1 μg of *E. coli* heat-labile toxin B subunit and 0.1 μg or 0.01 μg of attenuated cholera toxin were used together, the adjuvant enhanced immune response evidently much higher than that exhibited with the same amount of attenuated cholera toxin alone. The result shows that the dose of attenuated toxin can be further reduced by the method in which the attenuated toxin is used together with another adjuvant.

Example 6 - Preparation of various attenuated toxins and enhancing effect thereof on the antibody production:

Diphtheria toxin and pertussis toxin was prepared according to a method described by the Kitasato Institute for producing diphtheria vaccine and pertussis vaccine ("Textbook of techniques for vaccine production," Kitasato Institute, 1986). Each toxin was attenuated in the same manner as shown in Example 2 and was adsorbed on aluminum gel and used in the subsequent experiments. In addition, commercial Staphylococcal α toxin (Sigma Co.) and enteritis vibrio thermostable toxin (Sigma Co.) were purchased and attenuated in the same manner as described in Example 2. The toxins were used without further modification. A recombinant mutant LTR(7)K (lysine residue is substituted for arginine residue at amino acid position 7 from the

N terminus) of *E. coli* heat-labile toxin was prepared by a method described by Komase et al. (K. Komase et al., Vaccine 16, 248-254, 1998). Thus five types of attenuated toxins were obtained. The effect of enhancing immunity associated with influenza vaccine was tested according to the same method as described in Example 3, except that the attenuated toxins prepared above were used at appropriate concentrations instead of the attenuated cholera toxin in Example 3. The result is shown in Table 3, where relative value of residual toxic activity of attenuated toxin (ratio of toxin attenuated versus the natural one) is also indicated.

The result shows that both adjuvants of the invention, whether the natural toxin or the recombinant mutant toxin, exhibit activity of enhancing immunity associated with influenza vaccine administered by the intranasal vaccination route.